FINAL REPORT

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DEVELOPMENT OF NOVEL PLANT HOSTS FOR
MOLECULAR FARMING APPLICABLE TO SASKATCHEWAN

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FINAL REPORT

AFIF Project No. 96000410

Project Title: Development of Novel Plant Hosts for Molecular Farming Applicable to Saskatchewan

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I. SUMMARY

This multidisciplinary project was funded with the goal of identification of host plant species that could be genetically altered to serve as "molecular factories" for the synthesis of novel/valuable in industrial and/or pharmaceutical products in specific plant tissue/organs; these would then be extracted, processed and ultimately marketed as pure (chemical) products. The project aimed to develop this technology either in plants/crops that have traditionally not been grown as food crops or in biannual root crops that do not flower during the period in which they are harvested. In this way regulatory/public concerns regularding intermingling with food products or contamination by cross pollination are alleviated thus making this technology acceptable in commerce. The experimental results from this project resulted in the development of reliable genetics transformation technology for the following seed bearing species: *Camelina sativa*, and *Eruca sativa* and the root-bearing species *Brassica napus* var *rapifera* (rutabaga), and *Amoracia aristicana* (horseradish). In parallel studies, seed and root specific promoters were identified. Once completely characterized such promoters could be employed to target new gene expression in either seeds or roots of host plant systems.

Since this project was initiated there has been increasing interest in the development of novel host systems. The project was, in many ways, "ahead of its time", and it should help place Saskatchewan-based research organizations into an excellent position to continue to build on the solid technological base established through AFIF support.

II REPORT

A. Project Rationale

1. Background:

Research in plant biology and biochemistry during the last 30 years has clearly demonstrated that plants are capable of synthesizing and storing a very wide range of chemical products (e.g., proteins, oils, complex carbohydrates, and a range of secondary metabolites). The rapid advances in plant biotechnology (particularly gene identification/isolation and transformation) during the last 10 years have made it feasible to "genetically engineer' plants to become molecular factories, i.e., to synthesize and accumulate chemical products such as therapeutic proteins, vaccines, precursors for polymers and a range of industrial enzymes. The term "molecular farming' has been coined in reference to this type of technology. This emerging field is receiving a great deal of scientific and industrial interest. The applicants of this proposal were involved in the organization of a conference on Molecular Farming in Crops in Saskatoon in 1997. This highly successful meeting was the first official conference on this subject and was attended by approximately 300 persons.

Canada has continued to be a leader in organizing international level conferences on molecular farming with major conferences held in London Ont., Aug. 1999 and Quebec City, March 2003.

In Canada, R & D activities relevant to molecular farming are in progress in both public and private sector organizations. There are at least three molecular farming companies active in Canada; i.e., SemBioSys Genetics, Medicago Inc., and Guardian Biotechnologies Inc. Public institutions engaged in plant molecular farming research include: AAFC (London), Univ. of Guelph, Univ. of W. Ontario, Univ. of Calgary, and NRC-PBI.

2. Issues/Challenges Relevant to Molecular Farming

At the time that the proposal was developed a number of challenges were identified. These remain relevant and include:

i. Limited availability of transgenic host plant systems that can be cultivated in Saskatchewan and which are clear of patent restrictions. For example, the existing patent protection on transformation methodology in Brassica crops is often viewed as a formidable barrier for using canola as a target crop.

ii. Synthesis/accumulation and recovery of desired product at levels sufficient for economic viability.

iii. Regulatory/societal concerns about using the large acreage conventional food/feed crops as hosts for molecular farming (e.g., concern about accidental contamination of food with pharmaceutical products); use of our key export crops (e.g., canola, wheat, barley, flax, peas, lentils, mustard) for a range of molecular farming applications could lead to Canada being penalized in terms of international trade.

iv. Regulatory/environmental concern about escape of genes (cross pollination/contamination of conventional commercial varieties of the same crop species as well as cross pollination with related wild species).

In fact the regulatory and societal concerns re: molecular farming have become more prominent within the last year. Much of this concern can be attributed to the fact that a transgenic corn seed produced by a U.S. molecular farming company (Prodigene) and carrying molecular farming products were found as contaminants in soybean seeds. The food and grocery industries now appear to be developing serious concerns regarding the use of food crops as molecular farming hosts. The US Grocery Manufacturers' Association has sent a document to the FDA expressing their views and concerns on molecular farming systems (attached as Annex 1). It is possible that in N. America, the use of food crops for molecular farming will be prohibited. The expressed concerns are a strong positive validation of AFIF's decision to fund this project.

3. Project Investigators

NRC/PBI Researchers:
R. Datla
W. Keller (Project Leader)
C.E. Palmer (Guest Scientist)
L. Pelcher
G. Selvaraj

AAFC Researchers:
R. Gugel (AAFC-Saskatoon)
S. Warwick (AAFC, Ottawa)

University of Saskatchewan Researcher:
G. Scoles

B. Technical Report

[**Project History:** A revised work plan with a total budget of 600K, (reduced from the original total request of 1.7M was submitted August 31, 1998 and quickly approved by the AFIF Board. The project was formally initiated on April 1, 1999. The project was formally initiated on April 1, 1999. Major progress reports were submitted December 31, 1999 and August 22, 2001.]

The report is organized according to the key project elements provided in the revised work plan. A copy of the revised proposal/work plan is attached as Appendix 2.

I. Germplasm Accession and Evaluation (R. Gugel, S. Warwick)

(1) Molecular Evaluation (S. Warwick)
Molecular characterization to estimate genetic diversity and genotyping was undertaken with four potential host plant systems. These included *Crambe* (38 accessions), non oilseed types of turnip *rapa*, *Brassica rapa* (75 accessions), Ethiopian mustard, *Brassica carinata* (94 accessions), and horseradish, *Armoracia rusticana* (20 accessions).

Crambe: The study of the *Crambe* species complex was completed and included morphological, cytological and a range of molecular marker data (cpDNA, RAPDs and rDNA sequence data), and data from a 2-year agronomic field trial in Saskatchewan. This work was published in "Genetic Resources and Crop Evaluation" (a copy of this manuscript had been attached to the progress report submitted Aug. 22, 2001).

Brassica rapa: Molecular characterization studies were completed using RAPDs (random amplified polymorphic DNA). A poster was presented at the Cruciferae Genetics Workshop (Brassica 2000, Wellsbourne, UK in September 2000 and published in Eucarpia Cruciferae Newsletter 25: 13-14 (2001).

Brassica carinata: Preliminary work evaluating the utility of AFLPs for extracting levels of genetic diversity amongst *B. carinata, B. juncea,* and *B. nigra* and for accession identification in *B. carinata* has been conducted. A poster (by Warwick and Soleimani) was presented at the Cruciferae Genetics Workshop, Brassica 2000, Wellsbourne, UK and published in Eucarpia Cruciferae Newsletter 23: 15-16 (2001).

Amoracia: AFLP data analysis of the accessions were completed and data will be combined with agronomic data from Manitoba (Campbell Davidson, AAFC, Morden) for publication.

(2) Field Evaluation (R. Gugel)
Note: Field evaluations were conducted during the summers of 1998 (not covered specifically by AFIF), 1999 and 2000.

Accessions of *Crambe abyssinica* (57), *C. hispanica* (30), *C. glabrata* (7), *Eruca sativa* (161), *Raphanus sativus* (11), and *Brassica carinata* (95) were obtained from the AAFC-SRC collection and from genebanks in the USA, Canada, and Europe. Seed of these accessions was provided to Dr. S.I. Warwick, AAFC-ECORC, for molecular characterization. Seed of selected *Crambe* and *Raphanus* accessions was provided to Ms. G. Nowak, NRC-PBI, for transformation experiments.

All accessions were increased in isolation tents and evaluated in field tests at the AAFC-SRC Research Farm. Accessions that were misidentified were correctly identified to species based on morphological traits. Notes were made on agronomic characteristics of primary interest to plant breeders including days to flower, days to maturity, resistance to lodging, and height. Other traits of interest were also noted, where applicable. Blackleg resistance evaluations were conducted on the *Crambe* spp. and *E. sativa* accessions, and collaborations with Drs. R.H. Elliott and J.J. Soroka, AAFC-SRC, were initiated to conduct preliminary investigations on resistance to flea beetles and root maggots in these species. Seed was harvested from each plot, dried, cleaned, and evaluated for seed quality.

The seed quality evaluation was conducted by Dr. J.P. Raney, AAFC-SRC, and included determinations of oil and protein content, glucosinolate content and composition of the seed meal, and fatty acid content and composition of the seed oil. While not a part of this project, determination of seed quality traits provided additional data that should be useful for selecting accessions for transformation. Seed of *C. abyssinica*, *C. hispanica*, *C. glabrata*, *R. sativus*, *B. carinata*, and *E. sativa* was provided to Plant Gene Resources of Canada (PGRC).

A poster entitled "Evaluation of *Crambe* and *Eruca* germplasm in western Canada" by R.K. Gugel, R.H. Elliott, J.J. Soroka, and S.I. Warwick was presented at the 11th International Crucifer Genetics Workshop, 03-07 October 1998, Ste-Adèle, Québec, Canada.

A summary of all the agronomic and seed quality data for the *Crambe* accessions was incorporated into a manuscript entitled "Genetic variation in the *Crambe abyssinica* - *C. hispanica* - *C. glabrata* complex" was published in "Genetic Resources and Crop Evolution". (This manuscript was attached to the progress report submitted on Aug. 22, 2001).

All the agronomic and seed quality data are available from the PGRC.

(3) Summary and Conclusions re: germplasm evaluation:

Observations on the agronomic performance of these species indicate that *C. abyssinica*, *E. sativa*, and *R. sativus* can be grown successfully as seed crops in western Canada. These three species have visually distinct seed/pods that are not likely to be mistaken for any other crop kind grown for edible seed in western Canada, a trait that is attractive for molecular farming applications that produce molecules of interest in seed. The crossability of *R. sativus*, with *Brassica* species may be an issue. *Eruca sativa* will need chemical inputs to control early season flea beetle feeding, as is currently done for canola. Based on field observations and reports in the scientific literature, plant breeding efforts directed towards disease resistance would be required to ensure sustainable production of these three species since each of them is susceptible to Alternaria black spot.

[**Note**: *Crambe abyssinica* had been previously cultivated on the prairies as a source of high erucic acid. One of the

agronomic problems associated with this crop was its sensitivity to black spot disease, R.K. Downey, personal communication.]

The other two *Crambe* species are unsuitable due to a more indeterminate growth habit and a tendency to shed mature pods while still flowering.

The *B. carinata* accessions were extremely late in maturing, which increases the risk of frost damage and also increases damage from insect feeding in late fall since this is the only green tissue available at that time. Since this project was initiated, significant advances have been made by AAFC-SRC in the development of earlier maturing *B. carinata* lines, acceptable for cultivation on the prairies and AAFC researchers have indicated that *B. carinata* could be developed as an industrial crop (via conventional breeding methods). A problem with *B. carinata* as a host system for molecular farming is its relatedness to cultivated canola and while interspecies cross pollination appears to occur at very low frequencies, it will likely represent a concern to regulators, i.e.: open field cultivation. Additionally, seed similarity could lead to accidental intermingling with canola seed.

There will be an ongoing future need to continue field evaluation potential hosts for molecular farming. We propose to continue such studies in cooperation with AAFC researchers and will seek other sources of support for these activities. Species that warrant attention include *Camelina sativa* (see below).

2. **Tissue Culture and Genetic Transformation Studies (W. Keller, E. Palmer, G. Scoles)**

This was a key component of the program, involving the development of transformation technologies in target species. (Example photos given in Annex 3).

(1) *Eruca sativa* (rocket)

Evaluation carried out by the Crop Development Center and R. Gugel (see above) have clearly indicated that *Eruca sativa* can be grown to maturity in Saskatchewan. While yields are lower than canola, the crop would, nevertheless, be suitable as a molecular farming host. [*Eruca* has not been grown as a vegetable oil and it is unlikely that it will be.] It is a distinct species with seed size similar to canola. While it is a member of the cruciferae, it does not hybridize in the field with canola.

A M.Sc. student project on the development of Agrobacterium-mediated transformation technology was initiated in 1997 (during the early stages of the development of this research proposal) and completed in 1999. The M.Sc. study was considered relevant to, and part of, the overall AFIF project with support coming from Univ. of SK, PBI and Dow AgroSciences Inc.

The M.Sc. project (completed by Ms. Susan Hughes) was based on a thorough evaluation of *Eruca sativa* seedling explants (i.e., cotyledonary petiole, hypocotyls). Hypocotyl explants were consistently the most responsive in terms of plant regeneration and gene insertion. Reliable transformation frequencies (of between 0.1 and 1% of cultured explants) were achieved using the gene which confers Liberty resistance. Approx. 10 selected lines of mature herbicide tolerant transgenics were grown to maturity in growth chamber conditions. These were self-pollinated to produce seed.

Eruca is a strong outcrosser (exhibiting significant level of self incompatibility) and therefore seed yield from self-pollinated transgenic lines were low and inbreeding depression was evident in seedling populations. The inbreeding depression represents a significant technological disadvantage in using Eruca as a molecular farming host. No additional transformation studies have been conducted since 2000. A manuscript for publication has been prepared (but not yet submitted).

(2) *Crambe abyssinica*

Germplasm for tissue culture and transformation studies was received from Richard Gugel (see above). More than 15,000 seedling explants (i.e. cotyledons, hypocotyls) were cultured in a wide range of culture media. Conditions were established for reproducible and high frequency plant regeneration (i.e., from more than 50% of the cultured explants). This is a novel finding that has not been reported in the literature. Our data is publishable but we have elected not to publish regeneration data at this point.

A range of Agrobacterium-mediated transformation studies were undertaken, however, for reasons that were not apparent, stable transformed lines could not be selected. It is possible that cells that gave rise to new shoots (plants)

were not readily accessible to, or infectable by, Agrobacterium. Transformation studies with *Crambe* were actively pursued from 1999 until early 2001 where increasing emphasis was placed on *Camelina* (see below). However, we will be initiating a new effort on *Crambe* transformation as part of PBI's internal core program for the upcoming three-year period (i.e., April 1, 2003 – March 31, 2006). It is noted that *Crambe* has a number of potentially desirable features as a potential host for molecular farming: it is self-compatible, can be grown on the prairies and has no history of cultivation as a food crop.

(3) *Camelina sativa*

This cruciferous species which is also referred to as "gold of pleasure" can be grown in Western Canada. It has been cultivated in Eastern Europe as an industrial oilseed and has been evaluated for specialty oil production in the northern U.S. [**Note**: we have assembled literature as various evaluation studies on this crop. We would be pleased to make this available upon request.]

A series of tissue culture and Agrobacterium co-cultivation experiments were undertaken and more than 20,000 seedling explants were handled. The seedling explants produced abundant callus and in cases where co-cultivation experiments were undertaken, virtually 100% of the callus cells were transformed. Shoot regeneration could be achieved from more than 5% of the cultured explants, thus giving very good frequencies of transformed shoots. We have experienced difficulties in obtaining good root formation and in establishing transgenic plants in the greenhouse. Within the last year, progress has been made in using modified hydroponic systems and several transgenic lines have been grown to maturity. We anticipate further improvements in methods for plant establishment (using PBI core resources) and testing for gene stability will be required and we plan to pursue such studies.

In summary, *Camelina* is regarded as a relatively easy species to transform and therefore will offer good potential as a host system for molecular farming.

(4) *Raphanus sativus* (oilseed radish)

Oilseed forms of radish have been cultivated in Eastern Europe for several decades and field evaluation (see above)

have indicated that this species can be cultivated on the prairies.

Oilseed radish breeding line SRS 100 was obtained from Richard Gugel (see above). An assessment of more than 6,000 seedling explants revealed that hypocotyl explants were the most responsive. Shoot regeneration was achieved consistently in less than 1% of the explants. The frequency is too low for effective transformation studies.

Radish is a close relative to canola and can be crossed with canola and other radish species. Additionally, it is an outcrosser with substantial levels of incompatibility. A decision was, therefore, been made not to pursue radish intensively as a host for molecular farming.

(5) Horseradish (*Amoracia rusticana*)
Horseradish is a vigorous perennial root crop which is winter hardy and grows well in prairie conditions. Small acreages are grown in Eastern Canada and the U.S.A. to supply the condiment industry.

It should be noted that some contract production of horseradish was established in Manitoba to supply a Winnipeg company engaged in extracting the enzyme, horseradish peroxidase, widely used as a clinical protein standard and reagent. [Unfortunately, this business was forced into bankruptcy due to price undercutting by foreign competitors.]

Horseradish thus offers significant potential as a root delivery system for molecular farming. [While it is a perennial, it can be managed by standard cultivation and herbicide treatment procedures.]

Tests with more than 10,000 leaf and stem explants of greenhouse-grown horseradish has shown that it is highly regenerable. In some tests 100% of the leaf explants produce shoots.

Agrobacterium-mediated transformation experiments revealed that transformants could be produced in 10% of the cultured explants, and has since been increased to approximately 20%. Transgenic lines were established in greenhouse conditions and were easily propagated by standard cutting procedures.

Horseradish has clearly demonstrated its potential use in molecular farming as it transforms readily and specific transgenic lines could be maintained indefinitely as cuttings.

We have not tested transgenic lines in the field but would be in a position to do so, if required, by a collaborator.

(6) Rutabaga

While rutabaga is indeed a Brassica crop, its biennial nature predicates pollen drift and cross pollination. The roots do not survive Saskatchewan winters. The very high yield of biomass (50-1000 tonnes/hectare) make rutabaga attractive for certain types of molecular farming products (e.g. water soluble enzymes). In comparison to horseradish, the rutabaga roots are easier to harvest.

More than 5,000 seedling explants have been co-cultivated, with hypocotyls being the most responsive. Transgenic shoots have been obtained at frequencies of 0.1%-1% of cultured explants.

The work on rutabaga was undertaken in the latter part of the project and thus we did not have time to optimize/improve the system and characterize transgenic plants in the greenhouse.

(7) Preliminary Research and Potential Future Studies on Development of Host Systems

While emphasis was originally placed on Cruciferous species, it is appropriate that other plant families also be considered in the development of alternative host systems for molecular farming. Using in-kind resources, PBI has initiated tissue culture and transformation studies on the following:

i) Woad (*Isetis tinctoria*). This cruciferous species produces a tap root that has been used for centuries in the production of dyes. Reliable plant regeneration has been achieved from leaf petiole explants.

ii) Cow Cockle. This member of the carophyllaceae produces large seeds (similar to canola). Preliminary tissue culture studies are in progress.

iii) Sugar beet. This crop produces large roots and while used in the food industry, it is not cultivated in Saskatchewan. Low frequency (less than 1% of explants) of plant regeneration have been obtained.

PBI will continue evaluation of potential new host systems.

3. Promoter Tagging and Gene Targetting/Expression Studies

(1) Isolation and Molecular Characterization of Developmental Specific Promoters [R. Datla, L. Pelcher, G. Selvaraj]

Efficient gene transfer methods have been developed and routinely used in number of plant species. In recent years, advances in number of research fronts led to the identification of several useful genes with diverse functions that have many potential biotechnological applications in plant genetic engineering research. Besides application of these advances in developing genetically improved crop varieties with superior agronomic traits, in recent years the field of molecular farming using plants as potential target/system is getting significant attention as an attractive alternative both from pharmaceutical and non profit health research initiatives. To achieve optimized expression for introduced genes and production of useful compounds in plants, it is important to have these genes expressed appropriately in a regulated manner to realize the intended benefits and also to avoid the undesirable affects. Thus, availability of appropriate tissue and developmental specific plant promoters (regulatory sequences) are critical for engineering useful genes for molecular farming applications in plants. In this part of the project, the primary objective was to identify and characterize promoters that are root, embryo, and seed specific, which could be subsequently be used to drive the expression of desirable genes specifically for example in roots and/or seeds of selected target species for applications in molecular farming.

a) Identification and expression analysis of developmental specific promoter tagged lines: As a source for identification of relevant useful plant promoters, we have used the GUS:NPT-II fusion gene based promoter tagged populations that were generated at PBI. Employing the tagged gene GUS based histochemical staining assays; we have identified several potential candidate lines that

showed expression in root, embryos, and seed coat. Further detailed characterization of the root line showed high expression through different stages of root development, and also in wounded tissues (Annex 4, Fig. 1). Similar detailed expression analysis in embryo expression line showed predominant strong staining in the embryos and at very low levels in some vegetative tissues (Annex 4, Fig. 2), whereas the seed coat line showed highly localized expression only in the seed coat (Annex 4, Fig. 3).

b) Isolation and molecular characterization of root, embryo and seed coat promoters: To isolate the respective regulatory elements that constitute the putative promoters from the selected root, embryo, and seed coat tagged lines, first we have determined the molecular organization of the TDNA insertion(s) in these lines. These studies revealed that in root expression tagged line contain three copies, the embryo tagged line one copy, and the seed coat tagged line one copy, and corresponding molecular restriction maps were constructed. Based on these results, genomic fragments that include the target T-DNA flanking sequences from each of these tags have been isolated and used in the construction of respective sub-genomic libraries. Molecular screens using the tagged probes identified the positive clones that contain putative promoter elements for each of the three T-DNA copies (~1–3kb) from root line and respective single T-DNA copies from embryo (~1.5 kb) and seed coat (~2.0 kb) lines. The nucleotide sequence for these isolated putative promoters have been determined and analysis of these revealed in addition to the presence of signature "TATA" box, also presence of potential regulatory modules with partial/some homologies to elements (e.g. hormonal, wound responsive) present in other plant gene promoters. To determine if these isolated putative promoter sequences confer similar expression specificities as observed in the corresponding original tagged lines, recombinant expression constructs were generated using GUS as reporter gene in combination with these promoter fragments, and introduced into transgenic tobacco plants (approximately 20 independent lines for each construct). The GUS expression patterns for each one of these promoter fragments in transgenic tobacco plants showed that: (a) for one of the three isolated putative promoter fragments from root expression line, the expression was observed in root, but with some what lower (50%) than the original line suggesting elements conferring some quantitative functions

likely present outside of the isolated fragment, and additional detailed studies are required to resolve these results; (b) the promoter isolated from the embryo expression line directed GUS staining in the developing embryos reminiscent of the original tag: (c) where as the putative seed coat promoter showed expression specifically in the inner seed coat. Taken together, the promoter studies of this project identified and characterized three potential promoters for use in plant molecular farming applications.

(2) Gene Product Expression Studies [L. Pelcher]
The work was initiated on April 1, 2000 as this represents a " downstream" component of the overall program.]

In general, relatively low yields and difficulties in extraction and purification limit the large-scale production of recombinant proteins in plants. The goal of our research is to address these problems by development of a model system based on selective secretion of recombinant proteins either from roots or from shredded leaves into a simple hydroponic medium. Either of these approaches may offer a simplified and more cost-effective method for isolating recombinant proteins and enzymes with a pharmaceutical application from a simple medium rather than from complex plant extracts. We have chosen adenosine deaminase as the model enzyme. As reported previously, we have expressed mouse adenosine deaminase (ADA) in *Brassica napus*, *Nicotinia tabacum* and *maize*. High levels of active enzyme can be found in most organs and tissues of the transgenic plants. Non-transformed plants appear not to produce endogenous ADA and, therefore, lack any detectable activity. The colorimetric method used to assay ADA activity is simple and sensitive enough to detect broad range of enzyme activities.

a) Secretion from Roots: In order to facilitate secretion into a hydroponic medium we produced an ADA fusion gene using the secretion signal (sig) from acid phosphatase (AP) a phosphate-scavenging enzyme known to be secreted from roots of Arabidopsis and Brassica spp. We have synthesized a 167 base pair DNA fragment coding for the signal sequence (MSSRSDLKIKRVSLIIFL LSVLVEFCYGG FT↓SDM) derived from *Arabidopsis* acid phosphatase. We have cloned this fragment in frame with the coding sequence of the *ada* gene to produce a fusion gene. The resulting plasmid construct has been transformed into an ADA$^-$ *E. coli*

strain and tested for activity. All constructs show high ADA activity. Therefore, we concluded that the addition of the AP signal peptide did not inhibit ADA activity. The ada-apsig fusion gene was cloned into pPBI524 and subsequently into pRD400 and introduced into *Agrobacterium tumefacians* MP90. Three *A. tumefacians*MP90/ada-apsig strains were used for *B. napus* cotyledonary transformation (#7-1-2, #9-5-6, and #11-10-10). In all, two thousand ninety-five *B. napus* cotyledons were inoculated in ten separate experiments. Putative transgenics were rooted and tested for the presence of the 35S-35S ada-apsig-nos-term cassette in the plant genome by preparing DNA extracts from leaf tissue and amplifying the target DNA sequence via PCR analysis. Those putative transgenics which tested positive for the DNA insert were then checked for the production of active enzyme through preparation of protein extracts from leaf tissue and using aliquots in an adenosine deaminase activity assay developed in our lab. Transgenic plants which tested positive for both the presence of the cassette and production of active adenosine deaminase enzyme were retained. From construct #7-1-2 twenty two plants were retained (PCR+, activity +), #9-5-6 gave no transformants that were positive for both DNA insert and activity, #11-10-10 at this point has generated four plants which were PCR+, activity+. Of the twenty-six plants testing positive for DNA insert and enzyme activity ten have been planted in soil and seeds collected.

For root secretion studies hydroponic cultures were prepared with six of the *B.napus*/ada-apsig transgenics which were tested prior to use for high production of enzyme in the leaf tissue. Control plants of *B. napus* WT and *B. napus*/ada which were grown in tissue culture and tested for presence of active enzyme were used as control plants. The hydroponic plants were grown in individual baskets filled with inert clay beads to hold plant roots, which were designed for hydroponic growth. The plant roots grew well and extended throughout the clay beads, through the basket bottom and sides within a few weeks. Aliquots were taken from the root medium and either measured directly (100ul samples) or 15ml samples were concentrated over Centricon 3K columns to 100ul and measured for the ADA activity. No excreted active adenosine deaminase activity was observed for any of the transgenic or control plants in the root medium. The medium was tested determine if the ADA activity was being

inhibited by spiking the medium with active enzyme. No inhibition was observed.

We also compared the amount of ADA activity in leaf tissue vs root tissue from hydroponically grown plants. On a fresh-weight basis, leaf tissue from both ada and ada-apsig transgenic plants exhibited on the order of 40X more ADA activity then did root tissues. Therefore the absence of secreted ADA activity found in the hydroponics medium may reflect the low level of enzyme synthesis in the roots rather than inefficient or non-functionality of the acid phosphatase signal sequence attached to the ADA.

b) Initiation of Hairy Root Phenotype in B. napus to Enhance Root Secretion: Several studies have demonstrated that high levels of protein secretion occur mainly from merstematic root tip regions (Trull & Diekman, 1998; Borisjuk et al., 1999; Haran et al., 2000). Infection of *Agrobacterium rhizogenes* leads to the induction of cell differentiation and hairy root development (Krolicka et al., 2001). These roots are characterized by an extensive growth, associated with lateral branching and excessive root hair development leading to significant increase in biomass. Infection of *B.napus* tissue with A. rhizogenes (A4) was carried out with the goal of increasing development of lateral roots and root biomass in transgenic plants. For the preliminary studies *B. napus* WT and *B. napus*/ada plants were used. Roots of plants grown in tissue culture were excised, inoculated with *A. rhizogenes* and transferred to media for callusing and shoot formation. Infected roots produced callus with green bud-like structures on root-budding medium and to date two shoots have regenerated and were transferred to shooting medium with several more potential shoots developing. Cut stems of cultured plants were also dipped in *A. rhizogenes* and transferred to co-cultivation media for callusing. Stems from *B.napus* and *B.napus*/ada , which were not infected with *A. rhizogenes*, were also transferred to co-cultivation media as controls. Callus formed on the cut stem ends of all plants transferred to co-cultivation medium and they were subsequently transferred to rooting medium. Both infected and noninfected pants from all treatments developed roots on rooting medium. The non-infected stems grew roots that extended throughout the agar matrix, but not on the surface. The plants that developed from *A. rhizogenes* (A4) infected stem ends developed roots within the agar matrix with a high

frequency of lateral root formation which extended over the agar surface and up the sides of the tissue culture jar and lid. The surface roots (plagiotropy) with extensive lateral roots and root hairs are indicative of "hairy root" produced by *A. rhizogenes*. Protein extracts are currently being prepared from the surface root mass obtained from A. rhizogenes inoculated B. napus/ada. These extracts will be tested to determine if the ADA activity is higher than that observed in corresponding root systems *B. napus*/ada which was not inoculated with. *A. rhizogenes*. If we observe that the root systems from *A. rhizogenes* inoculated *B. napus*/ada plants produce significantly higher levels of ADA than non-inoculated plants we will induce the "hairy root" phenotype in transgenic *B. napus*/ ada-apsig plants and assay for the secretion of ADA from the roots of hydroponically grown plants.

c) Secretion from Shredded Leaf Tissues: To further test the functionality of the acid phosphatase signal sequence attached to the ADA we studied secretion of ADA from shredded leaf tissue. The high amount of adenosine deaminase activity in the leaves as opposed to root tissue lead to the possibility of the recovery of the enzyme through secretion into hydroponic media from leaf tissue. To test this hypothesis leaves from the hydroponic plantsused for root secretion studies were placed in 10ml sterile media in sterile dishes and sliced into 5mm strips with a scalpel. The shredded leaves were allowed to incubated for 24 hours and 100ul aliquots removed at 0, 1, 3 and 24hrs after slicing. The samples were then tested for activity. No significant ADA activity was observed in the solutions from either the *B. napus* WT or the *B. napus*/ADA samples over the 24hr period. In contrast, the shredded leaf samples from the two *B.napus*/ada-apsig transgenic lines an average average 22-fold increase in activity was over 24 hours indicating that the enzyme was being secreted into the medium. Based on activity as much as 25% of the total ADA was secreted into the hydroponic medium. It is possible that even higher amounts of secreted ADA could be achieved by finer shredding of leaf tissues.

We have demonstrated that root secretion signal sequences can be successfully exploited to direct the secretion of recombinant proteins (ADA-APsig) from shredded leaf tissues of transgenic plants (ada-apsig) of B. napus into simple hydroponic medium. These plants will be used to

further develop a model root secretion system for the continuous production recombinant proteins and enzymes with industrial or pharmaceutical applications.

C. Utility of the Information and Technologies to Saskatchewan: Biotechnology Industry

1. Guardian Biotechnologies Inc.

Guardian is a recently formed molecular farming company which is now located in PBI's Industry Partnership Facility. The President and CEO, Dr. Sun Lee received his doctoral degree from the Univ. of Sk in 1997, and had carried out his research in PBI. Guardian currently has four staff members and will grow to 10 within the next year. Ms. Goska Nowak, who was employed via AFIF funding in the development of tissue culture/transformation methodology, is now an employee of the company. Dr. Sun Lee has indicated that selection of the appropriate host systems is perhaps the key decision in the development of a molecular farming business plan. We intend to discuss our results and development mechanisms for technology transfer as Guardian's program evolves.

D. Personnel

Four researchers were recruited on a full time basis for periods of three years (in two cases), two years (one case), and one year (one case). The assignments were as follows:

i. Ms. Goska Nowak recruited (3 years) by W. Keller to undertake tissue culture and transformation studies.
ii. Dr. Venglat Prakesh recruited (3 years) by R. Datla to undertake promoter identification studies.
iii. Ms. Connie Hayes recruited (2 years) by L. Pelcher for gene expression studies.
iv. Ms. Tracey Macdonald recruited (one year) by S. Warwick for germplasm evaluation studies.

Other expenses related to personnel were the employment of a summer student (1999) for R. Gugel for field evaluation studies, and travel expenses for Prof. C.E. Palmer to undertake tissue culture/transformation studies in Saskatoon.

[**Note**: financial statements specifying salary and travel expenses have been previously submitted by PBI's Business Office.]

E. Equipment

No equipment was purchased with AFIF funds. All equipment requirements (laminar flow hoods, microscopes, centrifuges, PCR units, incubators, growth cabinets) were supplied as in-kind contributions by NRC and AAFC.

F. Project Photos

G. Innovation Fund Acknowledgement

The Innovation Fund has been acknowledged verbally and in written forms in conference/meeting presentations. We will continue to acknowledge the key role that this fund played in establishing this area of research.

H. Project Public Information Material

These have been attached to progress reports (including this report).

III GENERAL SUMMARY AND CONCLUSIONS

1. Reliable Agrobacterium-mediated transformation was achieved with two seed bearing species: *Camelina sativa* and *Eruca sativa* and five root-bearing species: *Brassica napus var rapifera* (rutabaga) and *Amoracia rusticana* (horseradish). While these species could serve as potential hosts in molecular farming, *Camelina* and horseradish are more responsive and would thus be viewed as priority species.

2. Excellent tissue culture and plant regeneration resuits were obtained with Crambe. This species could not be transformed with the procedure we employed, but because of its potential value as a molecular farming host, work will be continued as part of NRC's future research. Other potential hosts will also be evaluated in these future studies.

3. A number of reports and manuscripts derived from this work have either been published or are in various stages of preparation. We have not published transformation procedures as we view them as potential future IP or "know how" in terms of collaboration with industrial partners (see 4 below).

4. To date, no public service invention reports have been submitted and no formal protection actions have been initiated. However, it is possible that broader-band platform technology patents may be developed from aspects of this work.

5. Designated milestones involving field testing were not met. This project was very ambitious and insufficient labor was available to produce enough seed for field analyses.

6. Very good progress was achieved in isolating seed and root specific promoters. These have re-introduced into test systems (i.e., tobacco) and were shown to be functional. PBI is currently considering patenting groups of promoters and could be in a position to license promoters for molecular farming companies.

7. We have initiated dialogue with potential industry partners and in particular a local molecular farming company (Guardian Biotechnologies Inc.)

8. This project has provided an important research and development base for Saskatchewan in terms of molecular farming technologies. NRC is committed to continuing this work as part of its core research program.